AMEA-nın Xəbərləri (biologiya və tibb elmləri), cild 70, №2, səh. 7-12 (2015)

‘*Candidatus* Phytoplasma solani’ Associated With Grapevine ‘Bois Noir’ Disease In Azerbaijan

I.M. Huseynova[1,2*], G.Sh. Balakishiyeva[1,2], A.Ch. Mammadov[1,2], J.-L. Danet[3,4], P. Salar[3,4], X. Foissac[3,4], J.A.Aliyev[1,2]

[1]Institute of Molecular Biology and Biotechnology, Azerbaijan National Academy of Sciences, 2A Matbuat Avenue, Baku AZ1073, Azerbaijan
[2]Institute of Botany, Azerbaijan National Academy of Sciences, 40 Badamdar Highway, Baku AZ1004, Azerbaijan
[3] INRA, UMR 1332 Fruit Biology and Pathology, Villenave d’Ornon, France
[4]University of Bordeaux, UMR 1332 Fruit Biology and Pathology, Villenave d’Ornon France
**E-mail: i_guseinova@mail.ru*

During surveys conducted in the most important viticultural regions, such as Absheron, Ganja and Guba, the white varieties of grapevine (*Vitis vinifera* L.) dislpaying the yellowing and red varieties with the leaf reddening and leaf rolling symptoms reminiscent phytoplasma infections were collected for molecular analysis. Polymerase chain reaction assays using phytoplasma universal primer pair R16F2/R16R1 followed by primer pair R16F2n/R16R2 in nested PCR confirmed the association of phytoplasmas with symptomatic grapevines. Restriction fragment length polymorphism (RFLP) analyses using RsaI, and TaqI restriction enzymes indicated that grapevine phytoplasma isolates detected in Azerbaijan are belonged to ‘*Candidatus* Phytoplasma solani’ strain (16SrXII group). To verify the RFLP results, all of the positive samples were subjected to Stolbur group specific nested-PCR test based on *stamp* gene amplification using the primer pairs Stamp-F/R0 and Stamp-F1/R1. Amplification of non-ribosomal St*amp* gene confirmed the results of RFLP analysis. This is the first report of the ‘*Candidatus* Phytoplasma solani’ causing grapevine ‘Bois noir’ disease in Azerbaijan.

Key words: *‘Ca. Phytoplasma solani’, ‘Bois noir’, grapevine, Gara shani, leaf reddening, leaf rolling*

INTRODUCTION

Grapevine phytoplasma diseases known with the general name of “Grapevine yellows” represent a collection of widespread diseases in grapevine displaying similar symptoms that are associated with molecularly distinguishable phytoplasmas. The most important diseases in the main viticultural areas of Europe and Mediterranian countries are “Flavescence dorée” (FD) and “Bois noir” (BN) (Bertaccini et al., 2014). FD which appeared in south-west France in the 1950’s, is caused by phytoplasmas of the ‘elm yellows’ or 16Sr- V taxonomic group and is transmitted by the leafhopper *Scaphoideus titanus* in the persistent-propagative manner. BN, whose symptoms are indistinguishable from those of FD, was also first reported from France, then from the most important viticultural areas of Europe. BN disease, caused by ‘Candidatus Phytoplasma solani’, transmitted in the persistent-propagative manner by the planthopper *Hyalesthes obsoletus*. Other hopper species may also act as vectors since the disease has been observed to spread actively in geographic areas where *H. obsoletus* does not occur (Belli et al., 2010). ‘*Candidatus* Phytoplasma solani’ affects a wide range of crops and wild plants in the Euro-Mediterranean area and associated with several diseases (Quaglino et al., 2013). However ‘*Ca.* P. solani’ has recently been detected in Azerbaijan in annual crops such as eggplant, pepper and tomatoes, also in declining cherry and common meddler trees (Balakishiyeva et al., 2010) however up to now there have not been any reports on grapevine phytoplasma diseases in Azerbaijan. The aim of this study was to detect and identify phytoplasmas associated with grapevine.

MATERIALS AND METHODS

Recently, an extensive surveys were conducted main grape-growing areas in Azerbaijan and grapevines showing symptoms suggesting phytoplasma infection have been observed in main grape-growing areas in Azerbaijan. During surveys the white berried varieties of grapevine with the yellowing and red varieties with the leaf rolling and leaf reddening symptoms were collected in August and September 2014. Different local and foreign grapevine varietes showing yellowing,

leaf reddening and leaf roll symptoms reminiscent of phytoplasma infection and healthy plants for each varietes as negative control were collected (Table 1). Total DNAs were extracted from 1g fresh leaf midribs of diseased and symptomless plants (as control) following classical CTAB extraction protocol (Maixner et al., 1995). The DNA concentrations were measured by a nanospectrophotometer.

Total nucleic acid extracts were tested by 16S-rDNA nested PCR with the universal primers for phytoplasmas R16mF2 / R16mR1 and R16F2n / R16R2 (Gundersen and Lee, 1996). PCR was performed in mixtures (50 µL total volume) containing 100 ng of nucleic acid, PCR Buffer 1X, MgCl2 2 mM, 1 µM of each primer, 200µL of dNTP mix, and 2 units Taq polymerase. Following conditions were used: denaturation at 94ºC for 1 min (94ºC for 2 min for the first cycle), annealing (hybridation) at 60ºC during 2 min (55ºC for second amplification in Nested PCR), and primer extension (elongation) at 72ºC for 3 min and 10 min in the final cycle. The PCR products (7 µL) were analyzed by electrophoresis in the 1 X TBE buffer troughs 1% agarose gel, stained with ethidium bromide, and DNA bands visualized using a UV transilluminator.

For taxonomic characterization of detected phytoplasmas, the products (1.25 kbp) of the Nested PCR, obtained with primer pair R16F2n / R16R2, were subjected to enzymatic restriction fragment polymorphism (RFLP) analysis, with AluI (Sigma) and TaqI (Sigma) restriction endonucleases. 20 µl the enzymatic digestion mixture contained 0.5 µl of each enzyme (5 U/µl), 2 µl of 10x buffer, 13.5 µl sterilized H2O and 4 µl of PCR product. Digestion with AluI performed by incubation at 37 ºC overnight, but with Taq I incubated during two hours at 65 ºC. Phytoplasma strains FD-70 (Flavesence doree phytoplasma, 16SrV-C), Stolbur-Moliere ('Ca. P. solani', 16SrXIIA) maintained at INRA Bordeaux in *Catharanthus roseus* were used as references. Digested PCR products were analyzed on 3% agarose gel electrophoresis and visualized by staining with ethidium bromide under UV.

Table 1. Grapevine samples collected from Ganja, Guba and Absheron regions

Sample ID	**Grapevine variety**	**Collected region**	**+ nested PCR 16S R16R2-F2n**
GR1.AZ	Cabernet Savignon (red berried)	Ganja	-
GR2.AZ	Cabernet Savignon (red berried)	Ganja	-
GR3.AZ	Cabernet franc (red berried)	Ganja	-
GR4.AZ	Cabernet franc (red berried)	Ganja	-
GR5.AZ	Gara kishmish (red berried)	Ganja	-
GR6.AZ	Gara kishmish (red berried)	Ganja	-
GR7.AZ	Gara kishmish (red berried)	Ganja	-
GR8.AZ	Saperavi (red berried)	Ganja	-
GR9.AZ	Mahmudu (red berried)	Ganja	-
GR10.AZ	Mahmudu (red berried)	Ganja	-
GR11.AZ	Gara kishmish (red berried)	Ganja	-
GR12.AZ	Ag muskat (white berried)	Ganja	-
GR13.AZ	Bayanshira (white berried)	Ganja	-
GR14.AZ	Bayanshira (white berried)	Ganja	-
GR15.AZ	Ag muskat (white berried)	Ganja	-
GR16.AZ	Cabernet franc (red berried)	Guba	-
GR17.AZ	Cabernet franc (red berried)	Guba	-
GR18.AZ	Grenache (red berried)	Guba	-
GR19.AZ	Grenache (red berried)	Guba	-
GR20.AZ	Bayanshira (white berried)	Guba	-
GR21.AZ	Gara shani (red berried)	Absheron	+
GR22.AZ	Gara shani (red berried)	Absheron	+
GR23.AZ	Gara kishmish (red berried)	Absheron	+
GR24.AZ	Gara kishmish (red berried)	Absheron	+
GR25.AZ	Ag shani (white berried)	Absheron	-
GR26.AZ	Ag meleyi (white berried)	Absheron	-
GR27.AZ	Ag meleyi (white berried)	Absheron	-
GR28.AZ	Saray sarigilesi (white berried)	Absheron	-
Stolbur-Moliere	'*Ca.* P. solani' reference isolate	France	+
PD	'Ca. P. pyri' reference isolate	France	+
FD70	"flavescence dorée" reference isolate	France	+
PS	Healthy periwinkle	France	-

Stolbur group specific nested-PCR test based on *stamp* gene amplification (Fabre et al. 2011) were performed using the primer pairs Stamp-F/R0 and Stamp-F1/R1.

RESULTS AND DISCUSSION

During the late summer and early autumn in 2014, symptoms reminissent grapevine phytoplasma diseases have been observed in several grape-growing areas, such as Ganja, Guba regions and Absheron peninsula, in Azerbaijan. Observed symptoms were restricted to the three major types: leaf reddening, leaf rolling, and leaf yellowing. In the surveyed regions, yellowing symptoms appeared on the white varieties of grapevine, whereas the leaf rolling and leaf reddening were observed on the red berried grapevine varieties (Fig. 1).

Total DNA from twenty eight grapevine leaf samples with the leaf rolling and yellowing for white varietes and red varieties with the leaf reddening symptoms also from two symptomless plants collected in September 2014 were extracted and obtained DNA extracts were tested by 16S-rDNA Nested PCR (Fig. 2).

While symptomless plants gave no amplification, positive results (1250 bp amplicons) were obtained from four samples of red berried varieties of grapevine. The positive samples were from Absheron peninsula and belong to "Gara shani" local red berried grapevine varietes.

For identification of detected grapevine phytoplasmas, all of the obtained 16S-rDNA Nested PCR products were subjected to overnight restriction with enzymes *Alu*I and *Taq*I (Fig. 3). As shown from the Figure 3, 16S-rDNA-RFLP patterns of *Alu*I and *Taq*I from all of the four grapevine samples gave the same profile that the Stolbur-Moliere '*Ca.* P. solani' reference isolate.


A) B) C) D)

Figure 1. Observed symptoms reminissent grapevine phytoplasma diseases.
A, B - red berried varietes, C,D – white berried varieties.

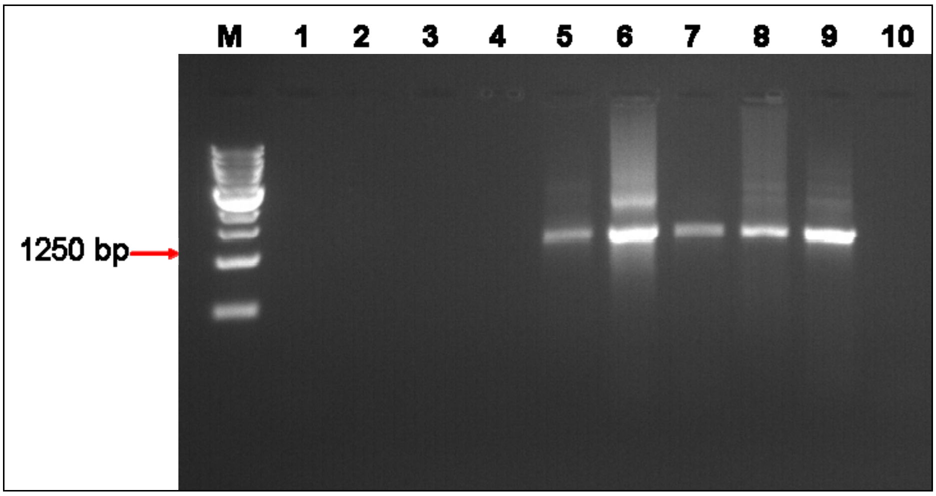
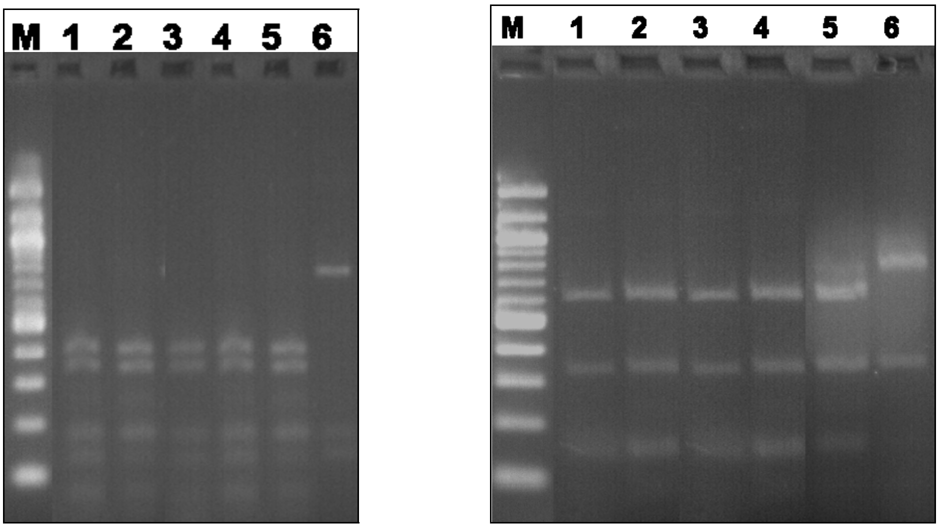


Figure 2. 16S-rDNA Nested PCR analysis with R16mF2 / R16mR1 andR16F2n / R16R2 universal praimer pairs for phytoplasmas. M- 1kb DNA marker (Sigma), 1-GR17-AZ; 2- GR18-AZ; 3- GR19-AZ; 4- GR20-AZ; 5-GR21-AZ; 6-GR22-AZ; 7-GR23-AZ; 8- GR24-AZ; 9- PD-positive control; 10-PS-negative control.



***Alu*I** **_Taq_I**

Figure 3. 16S-rDNA nested PCR-RFLP analysis. M-100 bp marker (Sigma), 1-GR 21.AZ; 2-GR 21.AZ; 3-GR 21.AZ; 4-GR 21.AZ; 5-St MOLIERE; 5-FD-70. Abbrevations were given in Table 1.

All positive grapevine samples were tested by '*Ca.* P. solani' specific nested-PCR based on *stamp* gene amplification (Fig. 4).

Nested PCR products were obtained for the four grapevine samples previously positives for 16S phytoplasma amplification. 'Ca. P. solani' was therefore recognized as the causal agent of grapevine diseases showing leaf reddening in Absheron region of Azerbaijan. '*Ca.* P. solani' has recently been detected in Azerbaijan in annual crops such as eggplant, pepper and tomatoes, but also in declining cherry and common meddlar trees (Balakishiyeva et al., 2010). *Hyalesthes obsoletus*, and a '*Ca.* P. solani'-infected cixiid planthopper are known to be present in Azerbaijan (Balakishiyeva et al., 2013). The 'Ca. Phytoplasma solani' was previously reported from such Middle Eastern countries as Lebanon (Choueiri et al., 2002),

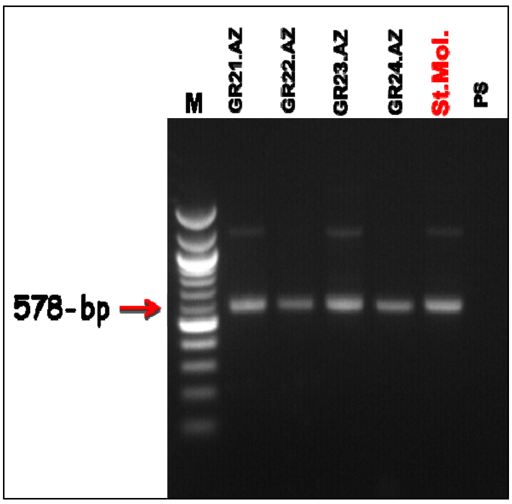


Figure 4. Nested-PCR based on *stamp* gene amplification.

Turkey (Canik et al., 2011), Jordan (Salem et al., 2013) and Iran (Mirchenari et al., 2015) as a causal agent of grapevine diseases, but up to present work there were no reports of phytoplasma diseases associated with grapevine in Azerbaijan. It is the first report of grapevine phytoplasma diseases and the first report of '*Ca.* P. solani' associated with grapevine BN disease in Azerbaijan.

ACKNOWLEDGEMENTS

This work was supported by the Science Development Foundation under the President of the Republic of Azerbaijan (**Grant № EIF-2013-9(15)-46/28/3).**

REFERENCES

Balakishiyeva G., Danet J.-L., Qurbanov M., Mamedov A., Khey-Pour A., and Foissac X. (2010) First report of phytoplasma infections in several temperate fruit trees and vegetable crops in Azerbaijan. *Journal of Plant Pathology,* **92 (S4):** 115.

Balakishiyeva G., Danet J.-L., Mammadov A., Huseynova I. and Foissac X. (2013). Survey of 'Candidatus Phytoplasma solani' and its potential vectors in Northern regions of Azerbaijan. *3rd European Bois Noir Workshop,* Barcelona: 56-58.

Canik D., Ertunc F., Paltrinieri S., Contaldo N., Bertaccini A. (2011) Identification of different phytoplasmas infecting grapevine in Turkey. *Bulletin Insectology,* **64 (Supplement):** 225–226.

Choueiri E., Jreijiri F., El Zammar S. (2002) First report of grapevine "Bois Noir" disease and a new phytoplasma infecting solanaceous plants in Lebanon. *Plant Disease,* **86 (6):** 697.

Salem N.M., Quaglino F., Abdeen A., Casati P., Bulgari D., Alma A., Bianco P.A. (2013) First report of 'Candidatus Phytoplasma solani' associated with grapevine Bois noir disease in Jordan. p. 23-24. *COST Action FAO 807* F*inal Meeting. Integrated Management of Phytoplasma Epidemics in Different Crop Systems.* Lisbon, Portugal, September 30–October 1, 85 pp.

Fabre A., Danet J.L., Foissac X. (2011) The stolbur phytoplasma antigenic membrane protein gene STAMP is submitted to diversifying positive selection. *Gene,* **472:** 37-41.

Gundersen D.E., Lee I.-M. (1996) Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathol Mediterr.,* **35:** 144-151.

Maixner M., Ahrens U., Seemuller E. (1995) Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology,* **101:** 241-250.

Mirchenari S. M., Massah A., Zirak L. (2015) 'Bois noir': new phytoplasma disease of grapevine in Iran. *Journal of plant protection research,* **55(1):** 88-93.

Quaglino F., Zhao Y., Casati P., Bulgari D., Bianco P.A., Wei W., Davis R.E. (2013) '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur and bois noir related diseases of plants. *International Journal of Systematic and Evolutionary Microbiology,* **63 (8):** 2879-2894.

Azərbaycanda 'Candidatus Phytoplasma solani' Fitoplazma Növünün Üzüm Bitkisində Törətdiyi 'Bois Noir' Xəstəliyi

İ.M. Hüseynova[1,2], G.Ş. Balakişiyeva[1,2], Ə.Ç. Məmmədov[1,2], J.-L. Danet[3,4], P. Salar[3,4], X. Foissac[3,4], C.Ə. Əliyev[1,2]

[1]AMEA Molekulyar Biologiya və Biotexnologiya İnstitutu
[2]AMEA Botanika İnstitutu
[3] Milli Kənd TəsərrüfatıTtədqiqatları İnstitutu, Fransa
[4]Bordo Universiteti, Fransa

Üzümçülüyün yaxşı inkişaf etdiyi Abşeron, Gəncə və Quba rayonlarına təşkil edilən fitopatoloji monitorinqlər zamanı yarpaqların saralması əlamətlərinə malik ağ və yarpaqların qızarması və burulması kimi fitoplazma xəstəliklərinin simptomlarını göstərən qara üzüm sortlarından nümunələr toplanılaraq molekulyar səviyyədə analiz edilmişdir. Fitoplazmalar üçün universal olan R16F2/R16R1 praymer cütlükəri ilə başlayıb nested PZR-də R16F2n/R16R2 cütlükləri ilə davam etdirilən Polimeraza Zəncirvari Reaksiyası

simptomatik bitkilərdəki xəstəliyin fitoplazma təbiətli olduğunu təsdiq etmişdir. *Rsa*I və *Taq*I restriksiya enzimləri ilə həyata keçirilmiş RFLP analizinin nəticələri Azərbaycanda üzüm bitkisində aşkar olunmuş fitoplazma izolyatlarının *'Candidatus* Phytoplasma solani' fitoplazma növünə (16SrXII qrupu) aid olduğunu göstərmişdir. RFLP analizinin nəticələrini yoxlamaq üçün bütün pozitiv nümunələr stamp geninin amplifikasiyasına əsaslanan və Stamp-F/R0 and Stamp-F1/R1 praymer cütlərindən istifadə etməklə stolbur qrupu üçün spesifik nested PZR testə məruz qoyulmuşdur. Qeyri-ribosomal *stamp* geninin amplifikasiyası RFLP anaizinin nəticələrini təsdiq etmişdir. Bu Azərbaycanda *'Candidatus* Phytoplasma solani' fitoplazma növünün üzüm bitkisində 'Bois noir' xəstəliyi törətməsi haqqında ilk məlumatdır.

Açar sözlər: *Ca. Phytoplasma solani', 'Bois noir', üzüm, Qara şanı, yarpaqların qızarması, yarpaqların burulması*

Болезнь Растений Винограда 'Bois Noir', Вызванная Фитоплазмой 'Candidatus Phytoplasma solani' В Азербайджане

И.М.Гусейнова [1,2], Г.Ш.Балакишиева[1,2], А.Ч. Маммадов[1,2], Ж.-Л. Данет[3,4], П.Салар[3,4], З. Фоиссак[3,4], Дж. А. Алиев[1,2]

[1]*Институт молекулярной биологии и биотехнологии НАНА*
[2]*Институт ботаники НАНА*
[3]*Национальный институт сельскохозяйственных исследований, Франция*
[4]*Университет Бордо, Франция*

Во время фитопатологических мониторингов, организованных в районы Абшерон, Гянджа и Губа, где хорошо развито виноградарство, были собраны образцы белых сортов винограда с признаками фитоплазменных болезней, таких как пожелтение листьев и черных сортов с признаками покраснение и скручивание листьев для проведения молекулярных анализов. В результате полимеразной цепной реакции с использованием универсальных для фитоплазмы парами праймеров R16F2/R16R1и нестед-ПЦР с парами праймеров R16F2n/R16R2 установлено, что болезнь растений с такими признаками имеет фитоплазменную природу. Результаты RFLP анализа, проводимые с рестриктазами *Rsa I* и *Taq I,* показывают, что обнаруженные в Азербайджане изоляты фитоплазм у растений винограда относятся к виду *'Candidatus* Phytoplasma solani'(16SrXII группа). Для подтверждения результатов RFLP анализа, все позитивные образцы подвергали нестед-ПЦР с использованием специфических для Stolbur групп Stamp-F/R0 и Stamp-F1/R1 пар праймеров для амплификации *stamp* гена. Амплификация нерибосомального *stamp* гена подтвердила результаты RFLP анализа. Эта первичная информация о болезни 'Bois noir' растений винограда, вызванной видом фитоплазмы *'Candidatus* Phytoplasma solani' в Азербайджане.

Ключевые слова: *Ca. Phytoplasma solani', 'Bois noir',виноград, Гара шаны, покраснение листьев, скручивание листьев*